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Abstract : Cross-species differences between human and fish estrogen receptor (ER) binding by environmental chemicals have been reported. To study ER transactivation in a fish cellular context, we stably co-transfected the PLHC-1 fish hepatoma cell line with a rainbow trout estrogen receptor (rtER) and the luciferase reporter gene driven by an estrogen response element (ERE). This new cell model, called PELN-rtER (for PLHC-1-ERE-Luciferase-Neomycin), responded to 17β-estradiol (E2) in a both concentration- and temperature-dependent manner, as well as to environmental ER ligands from different chemical classes: natural and synthetic estrogens, zearalenone metabolites, genistein, alkyphenoles and benzophenone derivatives. The comparison with other *in vitro* models, i.e. human reporter cell lines (HELN-rtER, MELN) and vitellogenin induction in primary cultures of rainbow trout hepatocytes, showed an overall higher sensitivity of the human cells for a majority of ligands, except for benzophenone derivatives which were active at similar or lower concentrations in fish cells, suggesting species-specificity for these substances. Correlation analyses suggest that the fish cell line is closer to the trout hepatocyte than to the human cell context, and could serve as a relevant mechanistic tool to study ER activation in fish hepatic cellular context.

Introduction

The widespread presence of endocrine disrupter compounds (EDCs) in the aquatic environment has become a very important issue of environmental concern over the past few decades, as these natural or man-made chemicals may cause adverse effects on wildlife (Sumpter, 2005). Given the complexity of the endocrine system as well as the diversity of chemicals and their modes of action, tiered approaches have been proposed for the screening (Tier 1) and testing (Tier 2) of EDCs (reviewed by Hotchkiss et al., 2008). Tier 1 includes both *in vitro* and short term *in vivo* assays. In this context, the evaluation of non mammalian *in vitro* screening assays has been clearly identified as an important need to be addressed in EDC testing strategies (Hotchkiss et al., 2008). However, compared to mammalian species, fewer non mammalian *in vitro* screening assays have been developed. The lack of species specific screening assay may represent an important gap in risk assessment of EDCs for aquatic organisms, and for fish in particular, since cross-species differences have been identified with regard to the molecular mode of hormone action (i.e. receptor binding affinities) or xenobiotic metabolism (Matthews et al., 2000; Wilson et al., 2007; Hotchkiss et al., 2008).

One important mechanism in EDC action is mediated by the modulation of estrogen receptor (ER) activation. Different assays exist to assess estrogenic activity of chemicals in fish. Among them, the most widely used is based on vitellogenin (VTG) induction in isolated fish hepatocytes (e.g. Pelissero et al., 1993, Smeet et al., 1999). Such *in vitro* assay is toxicologically relevant because it measures natural gene response in cultured cells derived from a main target organ of EDC (i.e. liver) and because it retains metabolic properties close to the *in vivo* situation. However, it has also some limitations for screening purpose since it is relatively time-consuming and may be the subject of inter-assay variability (reviewed by Navas and Segner, 2006). On the

other hand, the use of receptor mediated expression of stable reporter gene system using established cell lines serves as rapid, reproducible and specific assay. However to our knowledge, only few stable reporter gene assays using fish cell lines have been described (Ackermann et al., 2002), and none in hepatic cell context.

In this study, we describe the development of a new stable reporter gene assay for the assessment of ER activation by chemicals in fish cellular context, by using the PLHC-1 hepatoma fish cell line (Ryan and Hightower, 1994). In this model, kinetics of luciferase transactivation by estradiol as function of exposure duration and temperature were determined, as well as its activation by various ER ligands representative of different chemical classes. Finally, the comparison of this new *in vitro* model with other well established assays for estrogenicity assessment, namely VTG induction in isolated rainbow trout hepatocytes and human reporter cell lines derived from HeLa (HELN-rtER) and MCF-7 (MELN) cells, highlighted some response specificity, possibly linked to the fish receptor and/or fish cell context.

Materials and methods

Chemicals, materials and reagents

17β-estradiol (E2, CAS#50-28-2), 17α-Ethinylestradiol (EE2, CAS#57-63-6), Estrone (E1, CAS#53-16-7), Estriol (E3, CAS#50-27-1), 2,4-dihydroxybenzophenone (BP1, CAS#131-56-6), 2,2',4,4'-tetrahydroxybenzophenone (BP2, CAS#131-55-5), 2-hydroxy-4-methoxybenzophenone (BP3, CAS#131-57-7), 2,4,4'-trihydroxybenzophenone (THB, CAS#1470-79-7), diethylstilbestrol (DES, CAS#56-53-1), Hexestrol (Hex, CAS#84-16-2), genistein (Gen, CAS#446-72-0), 4-*tert*-octylphenol (4OP, CAS#140-66-9), 4-nonylphenol (4NP, CAS#54181-64-5), bisphenol A (BPA, CAS#80-05-7), α-zearalenol (α-ZEE, CAS#36455-72-8), β-zearalenol (β-ZEE, CAS#71030-11-0) and α-zearalanol (α-ZEA, CAS#26538-44-3) were purchased from

Sigma-Aldrich (France). All compounds were of purity higher than 98 %. Stock solutions of chemicals were prepared in dimethyl sulfoxide (DMSO) at 10 mM and stored at –20°C. Fresh dilutions of test chemicals were prepared before each experiment. Culture medium and additives were purchased from Gibco (France), fetal calf serum (FCS) and D-luciferin from Sigma-Aldrich (Quentin Fallavier, France). Cell culture plastics were obtained from BD Bioscience (France), except 96-well plates which were purchased from Greiner (France).

Plasmids

The construction of ERE-βGlob-Luc-SVNeo and pSG_5-$rtER_S$-puro plasmids that encode respectively for the luciferase reporter gene and the rainbow trout estrogen receptor short form (rtERα), has been described previously by Balaguer et al. (1999) and Molina-Molina et al. (2008), respectively.

PLHC-1 cell line : culture conditions and stable transfection

The PLHC-1 cell line, obtained from the American Type Culture Collection (ATCC CRL 2406), is derived from the hepatocellular carcinoma of the topminnow *Poeciliopsis lucida* (Ryan and Hightower, 1994). PLHC-1 cells were routinely cultured at 30°C in minimum essential medium with Earle's salts (E-MEM) supplemented with 10% v/v decomplemented fetal calf serum (FCS), 1% v/v non-essential amino acids, 1% v/v of sodium pyruvate, 50 U/ml of penicillin and streptomycin antibiotics in a 5% CO2 humidified atmosphere. For stable transfection experiments, PLHC-1 cells were plated onto 100 mm diameter Petri dishes in complete E-MEM without antibiotics. Twenty four hours after plating, confluent cells were co-transfected with the two plasmids described above by using the Lipofectamine 2000™ reagent (Gibco, France), according to the manufacturer's instructions. After three hours, transfection reagent was removed and cells were allowed to recover for 24 hours before addition of 3 mg/ml G418 and 0.5 µg/ml

puromycin as selecting agents. Medium was renewed every two days during one month before first clones were isolated and amplified. Only few resistant clones (about forty clones in three transfection dishes) were developed on the plates after one month of selection treatment with antibiotics. Nevertheless twenty clones could be isolated and tested for luciferase induction by E2. Among them, the clone 1.1 showed the highest induction of luciferase activity by E2. This clone was chosen for further experiments and called PELN-rtER for PLHC-1 ERE-Luciferase Neomycin-rtER.

Luciferase induction assay

PELN-rtER cells were seeded on 96 well plates (50 000 cells per well) in phenol red free medium supplemented with 3 % dextran-coated charcoal treated FCS to remove serum steroids (DCC medium) and left to incubate for 24 h before chemical exposure. This medium was used to avoid interference due to estrogenic activity of phenol red and serum steroids in the assay. Solvent (DMSO) content did not exceed 0.1 % v/v in the culture medium. Cells were exposed to test chemicals for 48 hours at 25°C. Luciferase activity was then determined in living cells as follows. The culture medium was removed and replaced by 50 µl of D-luciferin 0.3 mM in DCC medium. After 5 min allowing a stabilisation of the luminescent signal, luminescence counts were determined in a microplate luminometer (µBeta, Wallac). Results were expressed as percentage of maximal luciferase induced by E2, the reference ligand.

Vitellogenin assay in primary culture of rainbow trout hepatocytes (PRTH)

Adult male rainbow trout (*Onchorynchus mykiss*) were obtained from a local hatchery (INRA, Gournay-sur-Aronde, France). Fish were kept in tanks with aerated charcoal filtered tap-water at a temperature of 15 °C. Rainbow trout were fed with commercial fish food and acclimatized to laboratory conditions for a minimum of 2 weeks before use in the experiments. Hepatocytes

were isolated as previously described (Laville et al., 2004) and seeded in 96 well Primaria™ microplates at a density of 5×10^5 cells per well and cultured at 15 °C in phenol red free Leibovitz-15 medium (L-15) supplemented with 5% DCC serum, penicillin and streptomycin (50 U/mL each) and 10 mM HEPES. Cells were left to incubate for 24 h before exposure to chemicals for 96 hours. Solvent content (DMSO) did not exceed 0.1 % v/v in the culture medium and half of the medium was renewed after two days with fresh medium containing the test chemical at the desired concentration. VTG quantification in extracellular culture medium was performed using a competitive enzyme-linked immunosorbent assay (ELISA) according to the method of Brion et al. (2002), using the AA-1 anti-salmon vitellogenin polyclonal antibodies (Biosense, Norway) and home-made standard VTG purified from E2-induced male rainbow trout (Brion et al., 2002).

Data analysis

A range of concentrations of chemical (0.01 nM to 1 µM for estrogens and zearalenone metabolites and 1 nM to 10 µM for the other chemicals) were tested in triplicate in each independent experiment. Data were expressed as mean value of relative luminescence units (RLU) ± standard deviation (SD). Dose–response curves were modeled by using the Regtox 7.5 Microsoft Excel™ macro (available at http://eric.vindimian.9online.fr/), which uses the Hill equation model and allows calculation of EC_{50}. Relative estrogenic potencies (REP) were determined as the ratio of 17β-estradiol EC_{50} to EC_{50} of the test chemical.

Results

Influence of temperature and exposure duration on luciferase induction by 17β-E2

The stable PELN reporter cell line was first examined for its ability to respond to the reference ER ligand E2 under different assay conditions. Since the functionality of rtERα has been shown

to be sensitive to temperature (Matthews et al., 2002), we first tested effect of E2 at different temperatures. As seen in Fig. 1a, the EC_{50} value of E2 was slightly lower at 22 and 25 °C (5 nM) than that observed at 30°C (7 nM) or 37°C (12 nM). In terms of fold induction, the luciferase signal was also affected by the temperature as luciferase appeared to be less inducible at 22°C, and to a lesser extent at 25°C, than at higher temperatures. Hence, by considering both the affinity of E2 to the rtERα and fold induction of luciferase, we chose to perform assays at 25°C in our experiments. Induction of luciferase was also dependent on exposure duration (Fig. 1b). In our experiments, we thus determined that a 48 h exposure was appropriate to detect maximal luciferase induction without affecting the EC_{50} of E2.

Ability of different ER ligands to induce luciferase mediated by rtERα in PELN-rtER cells

The figure 2 presents the ability of known ER ligands from different chemicals classes to induce luciferase in PELN-rtER cells. Overall, all examined compounds were able to induce luciferase with various transactivation profiles, in terms of both EC_{50} values (Table 1) and maximum luciferase response achieved at the highest dose examined (Fig. 2). The natural steroid estrogens E1 and E3 (Fig. 2a), as wells as the synthetic estrogen EE2 (Fig. 2b) behave as total agonists as they elicited maximal transactivation relative to E2. By contrast, partial transactivation curves were observed with the mycoestrogen α-ZEA, the phytoestrogen genistein (Fig. 2a) and the pharmaceuticals DES and hexestrol (Fig. 2b), as maximum transactivation was 60-70 %.

The industrial chemicals presented also different profiles in their potency and efficacy. The alkylphenols 4-OP and 4-NP (Fig. 2c) were weakly estrogenic in this system, as 45 % of transactivation was achieved at the highest tested concentration (10 µM). Bisphenol A behaved as a partial ER agonist. It significantly induced luciferase at a relatively low concentration (0.3 µM) but this induction was limited as it reached a maximum response of 35 % relative to E2.

Interestingly, among the different xeno-estrogens tested, benzophenone derivatives most efficiently induced luciferase in PELN-rtER cells (Fig. 2d), especially BP2 and THB that behaved as total agonists for the rtERα, while BP1 induced partial activation of luciferase at 10 µM and BP3 was found to be non active.

VTG induction by ER ligands in isolated trout hepatocytes

In order to compare our results with PELN-rtER with a well recognised *in vitro* fish system in our laboratory conditions, some chemicals were tested for their ability to induced vitellogenin in isolated rainbow trout hepatocytes (PRTH). As expected, the results summarised in Table 1 indicate that all tested compounds induced Vtg in a dose-response manner allowing EC_{50} determination and were ranked: EE2<E2<α-ZEA<DES<Hexestrol<Genistein<BP2<4-OP<THB<BP1.

Discussion

The stable fish reporter system (PELN-rtER) responded to a diversity of estrogenic compounds with different transactivation potency and efficacy (Fig. 2). In order to determine how this cell model compares with other established *in vitro* systems, the EC_{50} and relative estrogenic potencies (REP) were compared to values previously published using two other stable reporter gene systems, namely the HELN-rtER (for HeLa-ERE-Luc-Neo transfected with the rtER) (Pillon, 2005, Molina-Molina et al., 2008) and MELN (for MCF-7-ERE-Luc-Neo) cell lines (Balaguer et al., 1999, Pillon et al., 2005) (Table 1). These two models consist of human cell lines (HeLa and MCF-7 cells) that stably express the luciferase reporter gene under the control of the rtERα and the human ERα (hER), respectively. In addition, the ability of chemicals to induce VTG synthesis in primary cultures of rainbow trout hepatocytes (PRTH) has been evaluated and is also reported in Table 1. Overall, the absolute sensitivity to E2 and a majority of the tested

compounds varied among the different assays, and were ranked: PRTH<PELN-rtER<HELN-rtER<MELN. The relative estrogenic potency (REP) values allowed ranking of chemicals that was fairly similar in all assays. Nevertheless, Pearson's correlation analyses showed that the fish cell line was better correlated to HELN-rtER (r^2=0.91, n=9) and PRTH (r^2=0.90, n=9) than to MELN (r^2=0.80, n=9), suggesting a good adequacy between the different fish-based assays.

The higher sensitivity of the human MELN assay can be partly attributed to the known lower binding affinity of E2 for the rtER than for the hER (LeDréan et al., 1995, Matthews et al., 2000, Molina-Molina et al., 2008), due to divergences in the amino acid sequences of the ligand binding domain of these receptors (Pakdel et al., 2000). However, this loss of sensitivity is not systematically found as certain xeno-estrogens, like polychlorobiphenyls (Matthews et al., 2000), some alkylphenols (Olsen et al., 2005) or zearalenone and its derivatives (Le Guevel and Pakdel, 2001), have been shown to bind to and activate rtER at equal or lower concentrations than those required to active hER.

In the present study, the most significant inter-assay difference concerns the estrogenic activity benzophenone (BP) derivatives, which were almost equipotent in PELN-rtER and HELN-rtER cells, and much less active in the human MELN cells. The good estrogenic potency of BPs towards rtER has been already reported in mammalian cell or yeast-based assays (Kunz et al., 2006, Molina-Molina et al., 2008). Here, we report that in a fish cell line, these compounds still have a high estrogenic potency that is in the same order as that of natural ER ligands such as estriol (Table 1). Our results strengthen the recent view that such emerging aquatic pollutants present significant hazard to fish (Kunz et al., 2006) and present further evidence to support the use of appropriate species-related assays to investigate hormonal activity.

In addition, the different metabolic capacities of the cells may also have some influence on the sensitivity of the assays to detect estrogenic activity (Olsen et al., 2005, Bursztyka et al., 2008). HELN-rtER cells are derived from HeLa cells, which are poorly metabolically competent as compared to PLHC-1 cells. The latter have retained significant metabolic capacities including phase I and II biotransformation and efflux transporter proteins (Zaja et al., 2007), which may contribute to reduce the intracellular availability of chemicals for the receptors. In trout hepatocytes, which maintain substantial metabolic capacities in culture, the different tested chemicals were generally active at significantly higher concentrations than in cell lines, although the use of different endpoints (i.e. luciferase activation versus VTG detection) likely influenced the sensitivity of the response. Nevertheless, the observation that EC_{50} values in PELN-rtER were generally closer to PRTH than to HELN-rtER, could reflect, at least in part, different metabolic capacities in the assays and again a good agreement between the two fish cell models.

In summary, the establishment of a reporter cell line that stably expresses rtER-mediated luciferase within a fish hepatoma-derived cell context is reported for the first time. It is proposed that such tool is useful to identify species-specific responses, as shown with benzophenone derivatives. However, the lower sensitivity of the response to a majority of chemicals than in similar human-derived reporter cell lines could lead one to conclude that these cells have limitations for a chemical screening purpose since false negative may occur when assessing weak estrogenic compounds. Nevertheless, correlation with the well-recognised vitellogenin assay in PRTH cells supports the use of PELN-rtER cells to investigate estrogenicity in fish hepatic cell context. Furthermore, the metabolic capacities and in particular the high expression of functional aryl hydrocarbon receptor (AhR) in PLHC-1 cells make the PELN-rtER cell line a

relevant mechanistic tool to study ER/AhR interaction on receptor transactivation by environmental chemicals in a fish cellular context (Aït-Aïssa et al., in preparation).

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Figure legends

Figure 1. (a) Influence of temperature on rtER transactivation (expressed as percentage of maximal luciferase induction) by 17β-estradiol (E2) in PELN-rtER cells after 24 h of exposure. (b) Kinetics of luciferase induction (expressed as relative luminescence units or RLU) by 17β-estradiol in PELN-rtER cells at 25°C. Values are means ± SD of triplicates.

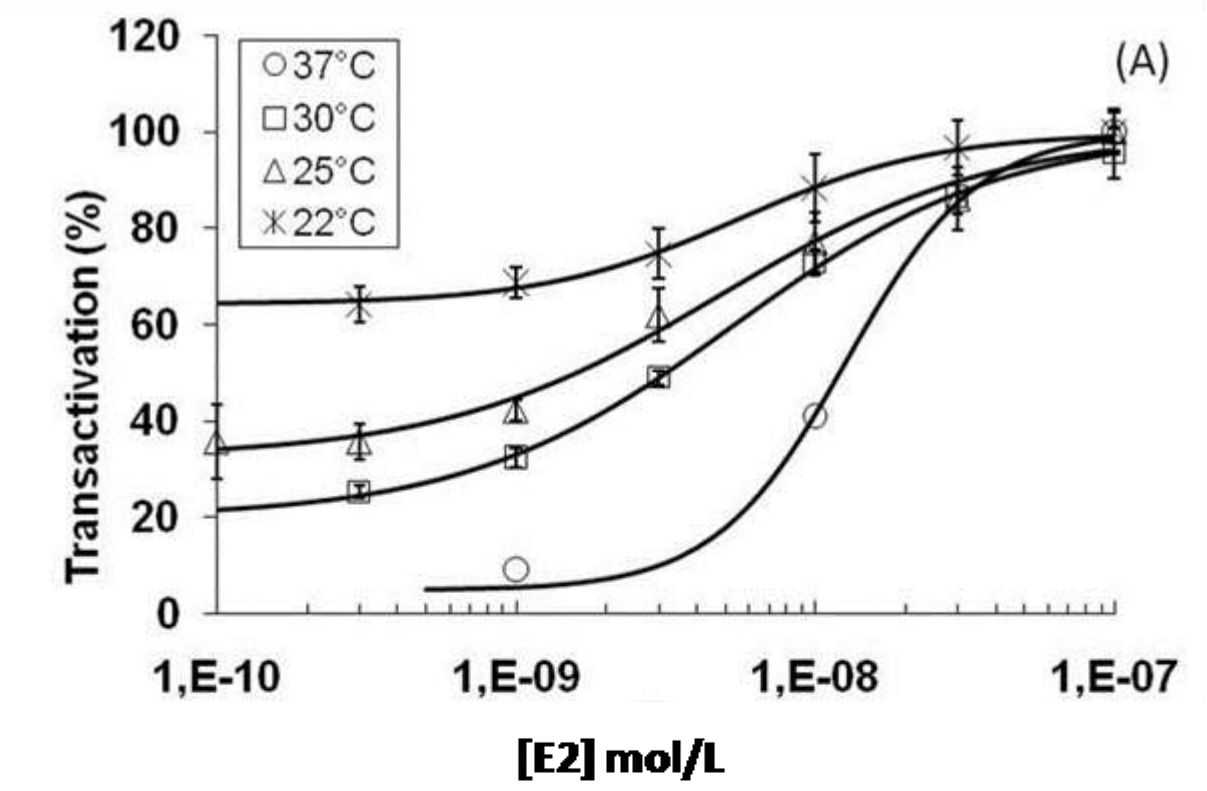
Figure 2. Typical dose response curves of luciferase induction (expressed as percentage of luciferase induction by E2 1 µM) in PELN-rtER by (**A**) natural (xeno)estrogens: 17β-estradiol (E2), estrone (E1), estriol (E3), α-zearalanol (α-ZEA), genistein (Gen), (**B**) pharmaceutical compounds: diethylstilbestrol (DES), hexestrol (Hex), 17α-ethynylestradiol (EE2), (**C**) alkylphenols: 4-nonylphenol (4-NP), 4-*t*-octylphenol (4-OP), bisphenol A (BPA) and (**D**) benzophenone derivates: 2,4-dihydroxybenzophenone (benzophenone 1 or BP1), 2,2',4,4'-tetrahydroxybenzophenone (benzophenone 2 or BP2), 2-hydroxy-4-methoxybenzophenone (benzophenone 3 or BP3), 2,4,4'-trihydroxybenzophenone (THB). Cells were exposed for 48 hours at 25°C; values are means ± SD of triplicates.

Table 1: Effective concentrations (EC_{50}) and relative estrogenic potencies (REP) of various ER ligands in fish (PELN-rtER, PRTH) and human (HELN-rtER, MELN) cell-based *in vitro* assays.

a: Except for benzophenone derivatives, HELN-rtER and MELN data were taken from Pillon (2005) and Pillon et al. (2005), respectively. Effect of benzophenone derivatives in MELN, HELN-rtER and PRTH were from Molina-Molina et al. (2008); *b*: PRTH data were obtained as described in the Materials and Method section.

n.d.: not determined ; n.a.: not active ; -: not applicable; *n*: number of independent experiments; SEM: standard error of the mean.

	PELN-rtER (fish PLHC-1 cells, rainbow trout receptor)				**HELN-rtER**[a] (human HeLa cells, rainbow trout receptor)		**MELN**[a] (human MCF-7 cells, endogenous receptor)		**PRTH**[b] (isolated rainbow trout hepatocytes, endogenous receptor)	
Chemicals	EC50 (nM)	*SEM*	*n*	REP	EC50 (nM)	REP	EC50 (nM)	REP	EC50 (nM)	REP
17β-estradiol (E2)	5.5	1.5	9	1	0.25	1	0.018	1	22	1
Estrone (E1)	130	25	3	0.04	12	0.02	0.69	0.03	n.d.	-
Estriol (E3)	176	58	3	0.03	7.8	0.03	0.10	0.18	n.d.	-
17α-ethynylestradiol (EE2)	3.6	0.4	3	1.54	0.18	1.39	0.01	2.56	12	1.94
Hexestrol (Hex)	11	5	5	0.50	0.2	1.25	n.d.	-	206	0.11
Diethylstilbestrol (DES)	23	10	5	0.24	0.8	0.31	0.18	0.10	130	0.18
α-zearalanol (α-ZEA)	26	14	5	0.22	0.12	2.08	0.14	0.13	41	0.56
α- zearalenol (α-ZEE)	47	-	2	0.12	0.25	1.0	n.d.	-	n.d.	-
β- zearalenol (β-ZEA)	504	-	2	0.011	2.5	0.1	n.d.	-	n.d.	-
Genistein (Gen)	498	-	1	0.010	220	1.1E-03	27	6.7E-04	1702	0.013
Bisphenol A (BPA)	352	83	3	0.016	400	6.3E-04	96	1.9E-04	n.d.	-
4-octylphenol (4-OP)	1938	-	2	2.9E-03	300	8.3E-04	54	3.3E-04	36271	6.3E-04
4-nonylphenol (4-NP)	23028	-	1	2.4E-04	600	4.2E-04	339	5.3E-05	n.d.	-
Benzophenone 1 (BP1)	3507	2515	3	1.6E-03	3477	7.2E-05	9192	1.9E-06	100000	2.3E-04
Benzophenone 2 (BP2)	384	142	4	0.014	161	1.6E-03	3284	5.5E-06	30000	7.6E-04
Benzophenone 3 (BP3)	n.a.	-	3	-	18426	1.4E-05	20315	8.8E-07	n.d.	-
Trihydroxybenzophenone (THB)	620	460	3	0.009	578	4.3E-04	4012	4.5E-06	60000	3.8E-04


(A)
37°C
30°C
25°C
22°C
Transactivation (%)
120
100
80
60
40
20
0
1,E-10
1,E-09
1,E-08
1,E-07
[E2] mol/L

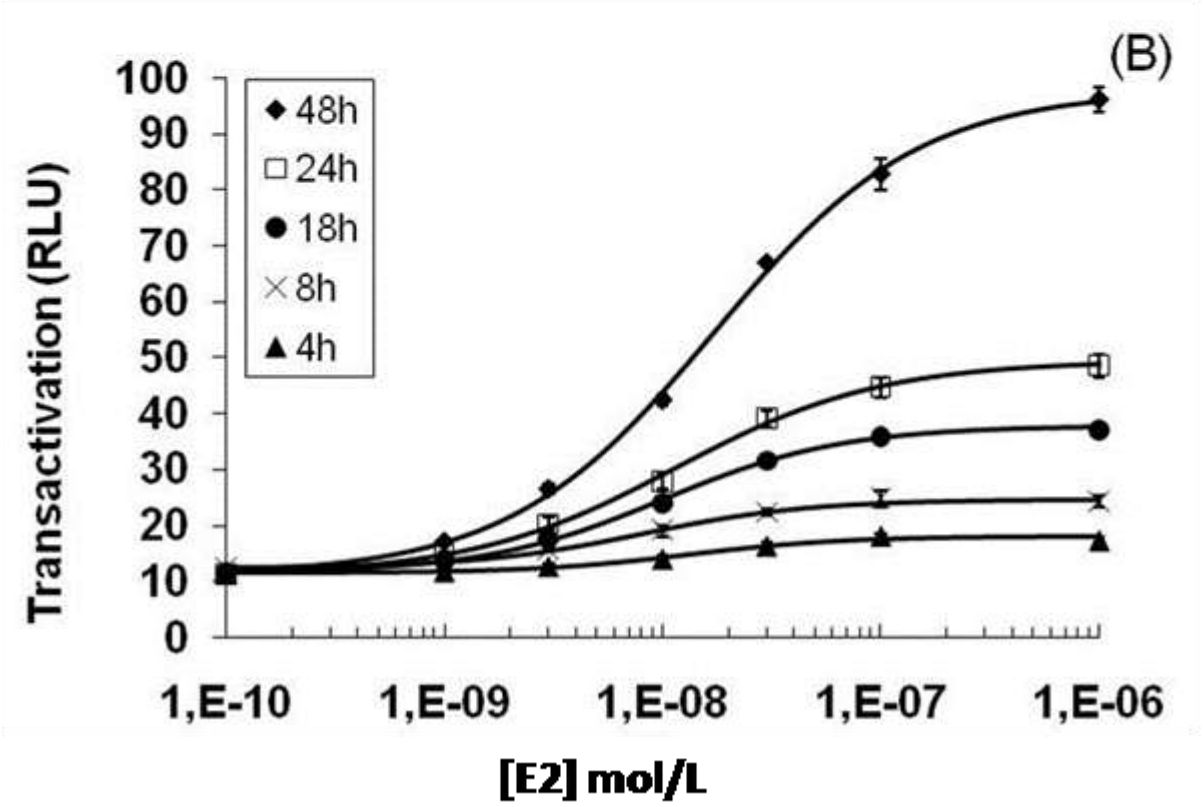

(B)
48h
24h
18h
8h
4h
Transactivation (RLU)
100
90
80
70
60
50
40
30
20
10
0
1,E-10
1,E-09
1,E-08
1,E-07
1,E-06
[E2] mol/L

Figure 1

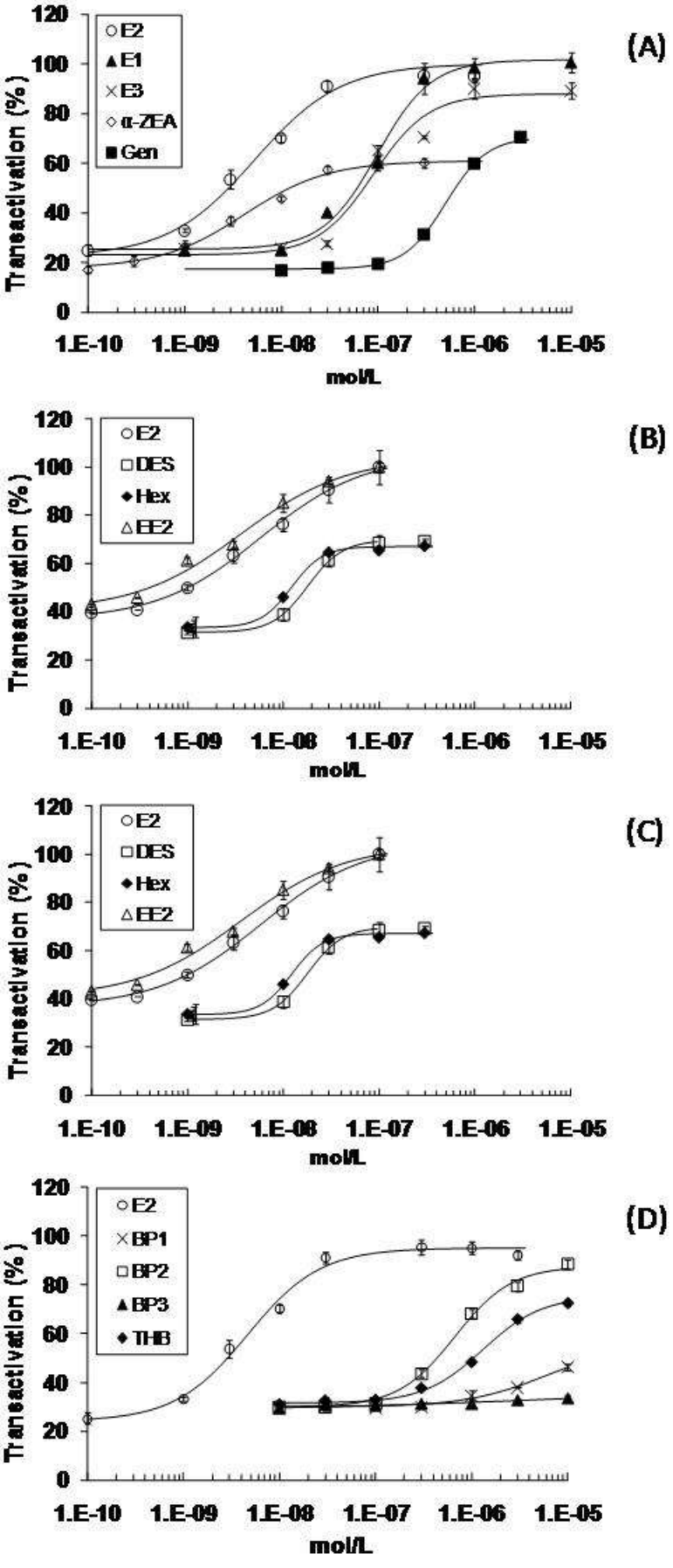

(A)
E2
E1
E3
α-ZEA
Gen
Transactivation (%)
mol/L
(B)
E2
DES
Hex
EE2
Transactivation (%)
mol/L
(C)
E2
DES
Hex
EE2
Transactivation (%)
mol/L
(D)
E2
BP1
BP2
BP3
THB
Transactivation (%)
mol/L

Figure 2